# ZEQUANOX™ - Environmentally Friendly Biological Control of Zebra and Quagga Mussels with *Pseudomonas fluorescens* Strain CL145A

## SUMMARY

**Marrone Bio Innovations (MBI) -**
**Commercial Partner**
**for Bacterial Biopesticide Discovered by Dan Molloy, PhD (New York State Museum)**

### Environmentally Friendly Mussel Treatment Has Advantages

Power generation facilities require annual maintenance and preventive programs to keep infestations of fouling zebra and quagga mussels (*Dreissena* spp.) in their cooling water intake systems under control. Currently it is necessary at many of these and other infested raw-water dependent infrastructures to administer controlled dosages of chlorine or other types of biocides for this purpose. Natural resource interest groups and regulatory agencies are reexamining the negative long-term use of biocides for this purpose. Both groups have made it clear that safe, non-chemical alternatives for controlling mussel fouling would be environmentally beneficial. Chlorination, for example, is a common control method, and when chlorine combines with organic compounds in water, potentially carcinogenic substances such as trihalomethanes and dioxins are formed (United States Environmental Protection Agency 1999; Thornton 2000). Should future regulatory actions result in the loss of chemical biocides, without an alternative control option, electric generation organizations and many other industries that rely on the withdrawal of surface waters for operational reasons are certain to experience economic penalties. These losses would be the result of decreased production brought on by increased facility maintenance and downtime. The availability of an equally effective, yet more environmentally benign, mussel control method to replace chlorine and other biocides is critically needed by power plants and other infested facilities.

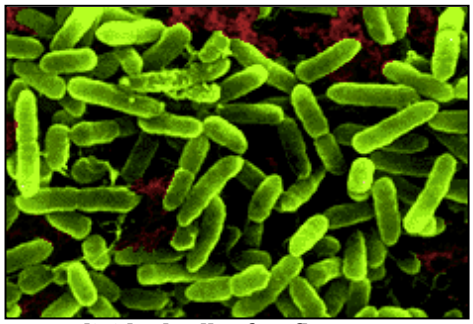
### Research Paradigm – Why Microorganisms?

It is widely accepted that the screening of diverse microorganisms and plant species for the natural products they produce is a worthwhile activity due to the discovery of drugs that can prevent or cure animal diseases, particularly cancers. More than 50% of today's drugs were derived from natural sources. Likewise, 11% of pesticides in use today are derived from plants and microorganisms. Therefore, it makes sense to look to microorganisms for unique natural products that have potential as highly selective biopesticides. In fact, the use of microbial natural product compounds already has a clear record of commercial success and environmental safety in the control of invertebrate pests in North America, as well as globally (Rodgers 1993). New York State Museum (NYSM) laboratory has been involved in such research for over two decades. Marrone Bio Innovations, the commercial developer of this mussel control product, has a team of scientists and a management team that have been involved in the biopesticide industry since its inception and are recognized leaders in "green chemistry" research, development and marketing. The MBI team has successfully introduced several biopesticide products to domestic and international markets over the past thirty years.

**Mussel Killing Bacteria**

Inception of Project – NYSM Screens Bacteria for Killing Zebra and Quagga Mussels

Faced with the threat of zebra mussels fouling electric power facilities within New York State, the Empire State Electric Energy Research Corporation (ESEERCO[1]) contracted with NYSM Field Research Laboratory in 1991 for the screening of bacteria as potential biological control agents. Extensive laboratory screening trials of more than 700 bacterial strains identified a North American isolate, strain CL145A of *Pseudomonas fluorescens*, to be lethal to these mussels. Of the strains of *P. fluorescens* that have been laboratory tested to date, only *Pf*-CL145A has been found to be highly lethal, i.e., at dosages that produce >90% adult zebra and quagga mussel kill and 100% larval kill with *Pf*-CL145A.



**Individual cells of *P. fluorescens*.**

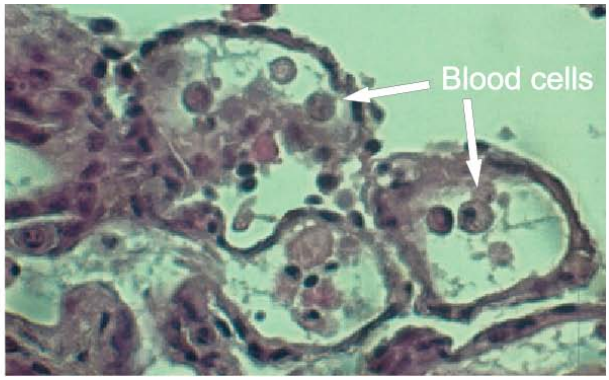
*Pseudomonas fluorescens* is worldwide in distribution and is present in all North American water bodies. In nature, it is a harmless bacterial species that is found protecting the roots of plants from plant diseases. NYSM research has shown that the *Pf*-CL145A strain of this species may be fortuitously used for another purpose — the control of *Dreissena* spp. (Molloy 2002). A patent for this purpose has been issued in both the United States (Molloy 2001) and Canada (Molloy 2004).

Mussels Die from a Natural Product: Dead Bacteria Kill Equally As Well

Although phytoplankton is their preferred food, *Dreissena* mussels can filter out and consume bacteria as a food source (Mikheev and Sorokin 1966; Frischer et al. 2000). When a zebra or quagga mussel ingests artificially high densities of strain CL145A, however, compounds within these bacterial cells destroy the mussel's digestive system. Dead cells are equally as lethal as live cells, providing clear evidence that the mussels die from natural compounds not from infection. Economical methods have been developed by MBI to kill the bacteria without any reduction in their lethality to the mussels. Findings from recent trials and bench tests indicate that MBI has significantly increased efficacy against zebra and quagga mussels. Commercial products developed by MBI based on this microorganism, which will be marketed as Zequanox®, contain dead cells, thus further reducing environmental concerns.

[1] A research consortium of New York State's electric power generation companies.

**In healthy mussels, epithelial cells (arrows) appear as a thick layer lining the tubules of the digestive gland.**




**Following bacterial treatment, epithelial cells are destroyed. Blood cells are abundant as the digestive gland hemorrhages.**

Mussel Feeding: Bacteria Are Readily Ingested

Although ingestion of CL145A cells is clearly a suicidal behavior for *Dreissena* mussels, they appear to have no adverse reaction to feeding on the cells and filter normally throughout a typical 6-hr, once-through pipe treatment. In contrast, biocides, like chlorine, that are currently being used for mussel control cause them to quickly shut their valves since the mussels apparently sense an adverse effect. This necessitates more prolonged chlorination periods, such as continuous treatments of three weeks or more. Unlike chlorine and other molluscicides, the acceptance of CL145A cells as "normal" bacterial food by these mussels facilitates the use of this microorganism as a biocontrol agent.

Mussel Length: All Mussel Sizes and Stages Can Be Killed; New Research on Veligers

All *Dreissena* mussel sizes tested to date (length, ca. 1-25 mm) appear to be equally susceptible to kill by CL145A. Thus, the bacteria are capable of killing, irrespective of mussel size. Susceptibility of the planktonic mussel stage has recently been the focus of NYSM research funded by the U.S. Bureau of Reclamation. Recent trials against zebra and quagga veligers show that this planktonic larval stage are more susceptible to the bacterial treatment that attached mussel stages. This indicates that bacterial treatments in open waters could significantly reduce veliger densities, thereby preventing high mussel density buildup and slowing the spread of mussel populations. It also suggests that very low doses of the biopesticide could be used to prevent veliger settlement within pipes and other water conveyances. Research into these initial observations continues.

**All mussel sizes show the same susceptibility.**

Mussel Species: Both Species Can Be Killed

Photo: NYSM

The bacterium can kill zebra mussels (*Dreissena polymorpha)* and quagga mussels (*Dreissena rostriformis bugensis*) — the two species that invaded North America in the 1980s. Lab tests at NYSM achieved higher kill against zebra mussels but there is excellent control of both species. New formulations developed by MBI have demonstrated commercial level kill at Davis Dam (US Bureau of Reclamation) of quagga mussels in multiple trials at doses even lower than those used originally at NYSM.

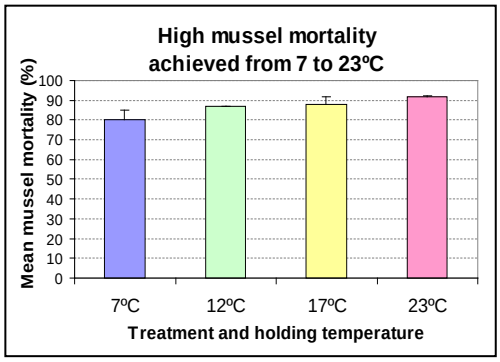
Water Hardness: Mussel Kill is Highest in Hard Water – the Preferred *Dreissena* Mussel Habitat

Tests to date at NYSM Field Lab suggest that bacterial treatments may have reduced efficacy in soft waters with pH values less than ca. 7.4. *Dreissena* mussels, however, rarely reach high population densities in such (near neutral or acidic) waters, and thus, infested pipes in power plants typically will have more alkaline waters where bacterial efficacy will not be impaired.

Dissolved Oxygen: Bacteria Work Well in Environments Where Mussels Thrive

Laboratory tests at NYSM indicate that very low oxygen levels (<2 ppm) can sometimes result in a 20% decline (e.g., 75% *vs*. 95%) in mussel kill. This is possibly due to lower feeding by the mussels on suspended bacteria under such low oxygen conditions. Thus, wherever possible, bacterial treatments should occur in waters of high dissolved oxygen – the preferred environment of *Dreissena* mussels.

Water Temperature: Higher Kill at Warmer Temperatures but Still Good Control in Cold Water



**Mortality increases with temperature.**

Research by the NYSM found that mussel susceptibility increases with water temperature, with >90% mortality consistently achieved in routine lab testing (at NYSM) at 23ºC against zebra mussels. High mortality is still achievable even in very cold waters, e.g., near 80% kill at 7°C, indicating that the bacteria are actually more effective at lower temperatures than currently commercialized chemical molluscicides used for *Dreissena* control. The latter commercial biocides, e.g., chlorine, can not achieve such high mussel kill below about 10ºC, thus limiting their application to warm water periods. At recent Davis Dam trials (January 2009), MBI showed commercial level of control of quagga mussels at Davis Dam at temperatures 10-14°C with new product formulations and application techniques.

Mussel Siphoning Behavior: Do Not Disturb Normal Mussel Feeding


In nature, a dreissenid mussel typically has its two shells spread apart and extends an inhalant siphon tube from between its shells to take food particles into its mantle cavity. After passing through the digestive system, fecal material is egested through the exhalant siphon. Testing has generally indicated that the more active this siphoning behavior is, the higher the mortality that will be achieved by a bacterial treatment. Thus, any stress factors (e.g., vibrations, shadows) that cause the mussels to close their shells during treatment will likely reduce mortality.

Treatment Concentration and Duration: Treat for no more than 6 Hours for Maximum Kill

Laboratory and facility trials conducted by NYSM and confirmed by MBI indicate that ca. 6-hr treatments of ca. 25-100 ppm (dry bacterial mass per unit volume) consistently obtain the highest mussel mortality. Exposing mussels to longer treatment durations or higher bacterial concentrations is (particularly >12 hr) achieves very limited further benefit. New formulations are currently being testing at both lower dose rates and shorter treatment times during the Spring and Summer of 2009. Jar tests in the US and Canada indicate that treatments of 2 hours or less may be effective in achieving commercially viable mussel mortality.

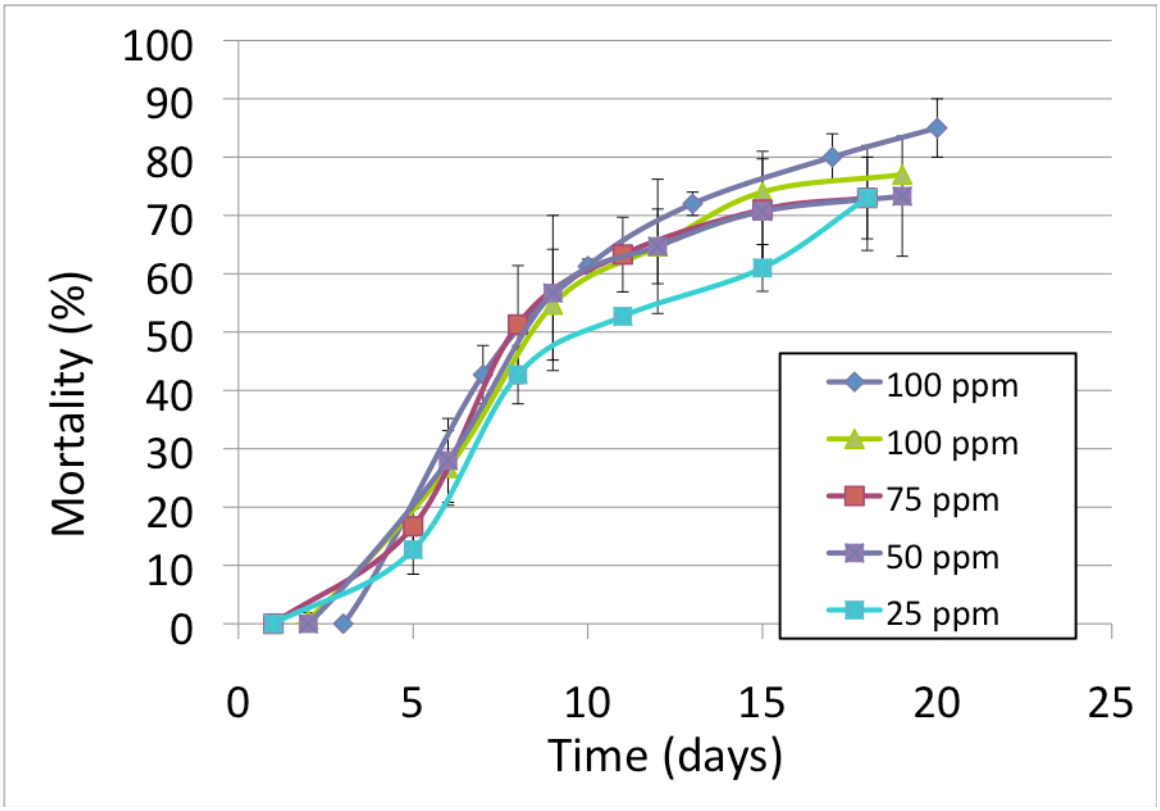
Trials at Power Plants: High Kill Can Be Achieved in Service Water

Previous NYSM-led trials routinely achieving high zebra mussel mortality (ca. 70–97%) in pipes have been conducted at the New York Power Authority (NYPA) electric power station on the Mohawk River (Crescent, New York). Trials at the Rochester Gas & Electric's Russell Power Station on Lake Ontario (Rochester, New York) have been primarily against quagga mussels (the less susceptible of the two North American *Dreissena* spp.) and routinely achieved 50-70% mortality.

MBI Trials at the U.S. Bureau of Reclamation's Davis Dam

MBI has conducted trials at the US Bureau of Reclamation's Davis Dam in fall 2008 and Jan-present 2009. The results of the trial show commercial level of control of adult quagga mussels. The trials were conducted in cold water (10-14°C). There will be even higher control in warmer water. The quagga kill is higher than that achieved by NYSM and this is likely due to improved formulation and application method developed by MBI.

The following chart shows the quagga mussel kill at different doses with a new formulation developed by MBI:




Additional trials are on-going at Davis Dam in Arizona, as well as companion trial at Ontario Power Generation's DeCew Hydro Facility in Canada, throughout 2009. MBI will be testing improved formulations that, in laboratory jar tests, have demonstrated greater than 90% mortality within 24 hours of treatment.

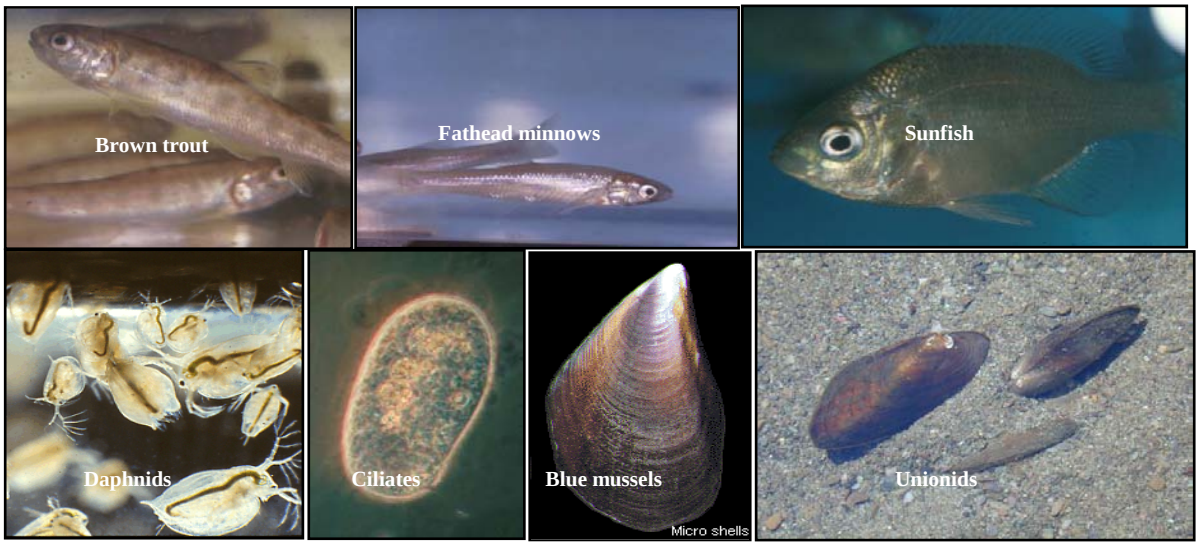
**Safety Testing**

Nontarget Organism Trials: Outstanding Specificity

Tests conducted to date have been very encouraging regarding nontarget safety. At dosages that produced high zebra mussel mortality (76–100%), no bacteria-induced mortality was recorded among any of the nontargets, including fish, ciliates, daphnids, and bivalves:

- Fish: No Pf-associated-induced mortality has been observed in the three fish species thus far tested: fathead minnows (*Pimephales promelas*), young-of-the-year brown trout (*Salmo trutta*), and juvenile bluegill sunfish (*Lepomis macrochirus*). Laboratory and facility trials have indicated that fish cannot tolerate exposure to high levels of live bacteria. Fish trials conducted with dead bacteria, however, have indicated that applications of killed cells were harmless to the fish, but yet were still highly lethal to the *Dreissena* mussels. Commercial products based on this microbe will contain almost exclusively dead cells.
- Ciliates: Trials with the common freshwater ciliate *Colpidium colpoda* indicated that the bacteria were not only nonlethal, but served as a food source permitting higher rates of ciliate reproduction than ciliates held in untreated streamwater.

- Daphnids: The microcrustacean *Daphnia magna* is an aquatic filter feeder that ingests small suspended particles including bacteria, making it an appropriate organism for non-target tests. Laboratory assays indicate that the bacteria are not lethal to this species.
- Bivalves: Bacterial exposures caused no mortality to blue mussels (*Mytilus edulis*) or any of six native North American unionid clam species (*Pyganodon grandis, Lasmigona compressa, Strophirus undalatus, Lampsilis radiata, Pyganodon cataracta,* and *Elliptio complanata*).




Studies With Freshwater Shrimp, *Hyalella azteca*

Experiments at the NYSM funded by MBI through a Phase II National Science Foundation STTR grant found no Pf-associated mortality and have indicated low risk to shrimp by the dead bacteria.

Studies With Mallard Duck

MBI contracted a study of the effect of the product against the mallard duck. The Pf-based biopesticide is nontoxic to mallard duck.

Acute Toxicology Studies to Assess Potential Human Health Risks

MBI has contracted acute toxicity (mammalian) tests required by the EPA and PMRA for product registration. The tests were conducted with living cells = the TGAI (Technical Grade Active Ingredient). Although the end product will be dead cells, tests against rats with the TGAI are required to assess worker exposure risks in manufacturing and handling. On skin and in feeding (oral) tests, and inhalation, there was no effect on the rats. The oral LD50 is >5000 mg/kg, which is the lowest risk category for pesticide products. Studies also demonstrate that the bacteria are non-pathogenic to rats.

## Additional Steps in the Commercialization Process

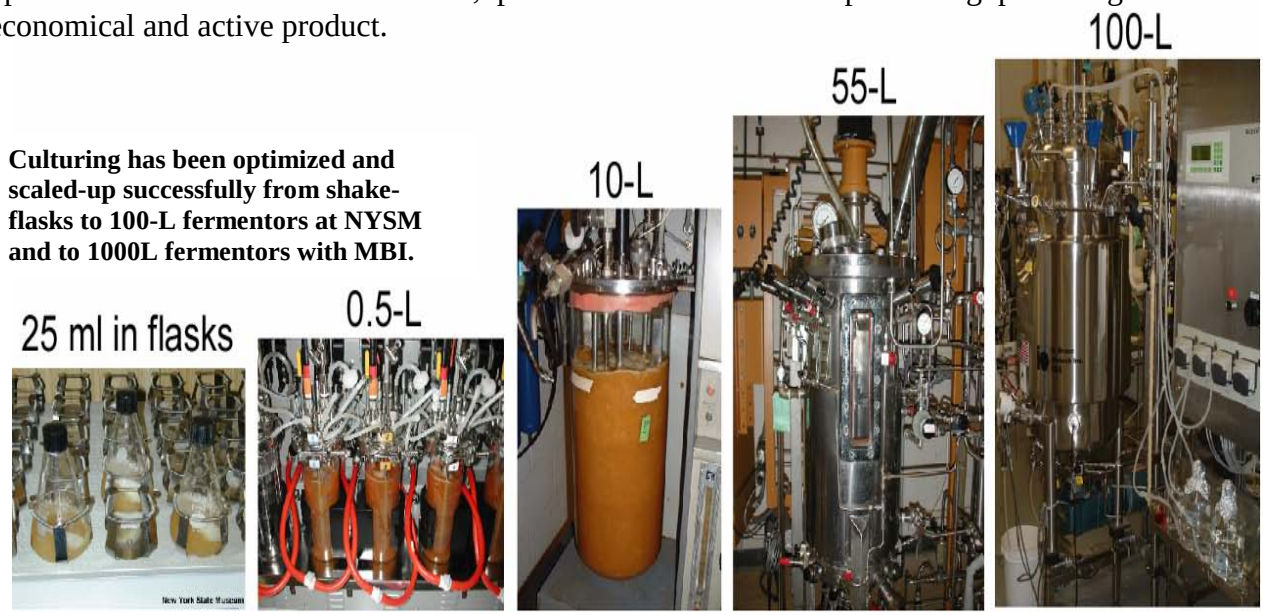
Identity of the Natural Product that is Lethal to *Dreissena* Mussels

Efforts have been underway at MBI to purify, characterize, and identify the natural product compounds produced by the *Pseudomonas* strain. MBI has identified and filed a patent on several unique compounds produced by the bacteria. There are more than 18 compounds in two different chemical classes that work together to cause activity. This complexity makes is less likely that mussels can develop resistance to the chemistry in this product.

Culturing Optimization and Fermentation Scale-up

*P. fluorescens*-CL145A cells are harvested during late linear to stationary growth phase under defined culture conditions. MBI has successfully scaled the product to 1000 L fermentation, and 10,000 L fermentation runs are scheduled for the Summer of 2009. Media components and fermentations parameters have been optimized by NYSM to result in a proprietary medium and protocol that consistently produce mussel-active cells. MBI has further optimized the fermentation medium, process and downstream processing providing a more economical and active product.

**Culturing has been optimized and scaled-up successfully from shake-flasks to 100-L fermentors at NYSM and to 1000L fermentors with MBI.**




Formulation

Under a phase II NSF STTR grant, MBI has developed an improved commercial formulation (liquid and spray dried powder) and is working on formulations for different market segments – open waters, utilities and water treatment plants

Additional Project Activities

The lack of non-target impact when treating with dead cells may allow this novel technology to also be used for *Dreissena* mussel control in open waters, such as lakes and rivers. Thus far, however, the research focus of the project has been controlling these mussels within power plant pipes. Other target markets are fish hatcheries, drinking water plants, other industrial plants and open waters. MBI will be conducting field trials in these markets and open waters as soon as resources allow.

Commercialization Partnership

Following a nation-wide search, the NYSM chose to partner with Marrone Bio Innovations (http://www.marronebioinnovations.com), a biopesticide company because its staff had unparalleled experience in the discovery, development, and marketing of natural products for pest management. The technology transfer under the grant is largely completed and MBI is rapidly commercializing the product after building on the NYSM knowledge base.

EPA Submission and Commercial Availability

MBI submitted the full Section 3 registration to the Biopesticide Division at the EPA in December 2008, with an anticipated registration during the Spring/Summer of 2010. The US Bureau of Reclamation is currently considering a Section 18 Emergency Use request for seventeen western states where the mussels are spreading rapidly and causing economic and environmental harm. MBI and Ontario Power Generation (OPG) recently received a Research Authorization and Certificate of Approval from Canadian regulators to conduct commercial-scale trials at OPG's DeCew II Generating Station this Summer. A dossier to register Zequanox is currently being prepared for submission to Canada's Pest Management Regulatory Agency.

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